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The Neurofibromatosis 2 Tumor Suppressor Gene Product, Merlin, Regulates Human Meningioma Cell Growth by Signaling through YAP[1]

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Abstract

Neurofibromatosis type 2 (NF2) is an autosomal dominant disorder characterized by the occurrence of schwannomas and meningiomas. Several studies have examined the ability of the *NF2* gene product, merlin, to function as a tumor suppressor in diverse cell types; however, little is known about merlin growth regulation in meningiomas. In *Drosophila*, merlin controls cell proliferation and apoptosis by signaling through the Hippo pathway to inhibit the function of the transcriptional coactivator Yorkie. The Hippo pathway is conserved in mammals. On the basis of these observations, we developed human meningioma cell lines matched for merlin expression to evaluate merlin growth regulation and investigate the relationship between *NF2* status and Yes-associated protein (YAP), the mammalian homolog of Yorkie. *NF2* loss in meningioma cells was associated with loss of contact-dependent growth inhibition, enhanced anchorage-independent growth and increased cell proliferation due to increased S-phase entry. In addition, merlin loss in both meningioma cell lines and primary tumors resulted in increased YAP expression and nuclear localization. Finally, siRNA-mediated reduction of YAP in *NF2*-deficient meningioma cells rescued the effects of merlin loss on cell proliferation and S-phase entry. Collectively, these results represent the first demonstration that merlin regulates cell growth in human cancer cells by suppressing YAP.

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Introduction

Neurofibromatosis type 2 (NF2) is a cancer predisposition syndrome phenotypically characterized by the occurrence of multiple nervous system tumors. The two most common tumors in this inherited syndrome are schwannomas and meningiomas [1]. Whereas meningiomas from individuals with NF2 exhibit biallelic inactivation of the *NF2* gene, loss of *NF2* expression is also detected in as many as 60% of sporadic meningiomas [2]. Similarly, genetically engineered mice with leptomeningeal *NF2* inactivation also develop meningiomas [3,4]. These findings strongly implicate the *NF2* gene in the pathogenesis of meningiomas; however, the molecular mechanism by which *NF2* regulates cell growth relevant to meningioma tumorigenesis remains unsolved.

Merlin (or schwannomin), the product of the *NF2* gene, is a member of the protein 4.1 family that links the actin cytoskeleton to plasma membrane proteins [5]. Although few studies have examined merlin loss in meningioma cells, loss of merlin in fibroblasts and Schwann cells results in loss of contact-dependent inhibition of proliferation, enhanced growth in soft agar and tumor formation in mice [6,7]. In these cell types, merlin has been implicated in epidermal growth factor receptor [8], β_1-integrin [9], and CD44 [7] function as well as Ras [10], Rac1 [11,12], phosphatidylinositol 3-kinase [13], mitogen-activated protein kinase [14], and signal transducer and activator of transcription [15] intracellular signaling. It is not known whether any of these growth control pathways are deregulated in *NF2*-deficient meningioma tumors.

Negative regulation of growth by merlin is conserved in *Drosophila*, where it acts upstream of the Hippo signaling pathway to coordinately

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regulate cell proliferation and apoptosis [16,17]. Mutations in merlin or other components of the Hippo pathway such as the serine/threonine kinases, Hippo and Warts, the adaptor molecule, Salvador, or Mats, results in activation of the transcriptional coactivator, Yorkie. Yorkie regulates expression of downstream target genes including *cyclin E* and *DIAP1* (*Drosophila* inhibitor of apoptosis protein 1) causing increased growth, delayed cell cycle exit, inhibition of apoptosis, and enhanced cell survival [16,17].

Individual components of the Hippo pathway are highly conserved in mammals, where they also regulate cell proliferation and apoptosis (Figure 1) [18,19]. Mice and humans have two Warts orthologs, Lats1 and Lats2. Mice deficient for Lats1 develop soft-tissue sarcomas and ovarian tumors [20]. The human ortholog of Salvador, hWW45, is mutated in cancer cell lines [21]. The two mammalian Hippo homologues, Mst1 and Mst2, promote apoptosis and regulate cell cycle exit [22]. Vertebrate Mst2 can rescue the lethality and overgrowth phenotypes of Hippo mutants in *Drosophila* [23]. Similar to their *Drosophila* counterparts, human Mst2 phosphorylates and activates both Lats1 and Lats2 [24]. The Yes-associated protein (YAP), the mammalian ortholog of Yorkie, is the primary effector of the mammalian Hippo pathway. Similar to the function of Yorkie in *Drosophila*, YAP causes aberrant tissue expansion in mice and induces epithelial transformation in mammary cells [25,26].

Given the conservation of components and mechanisms that operate downstream of merlin between *Drosophila* and mammals, we tested the functional relationship among merlin, Hippo pathway regulation, and growth suppression in human meningioma tumors. We developed nonneoplastic and neoplastic meningeal cell lines that mimic gain or loss of *NF2* expression and used these matched lines to examine merlin regulation of YAP. We found that absence of merlin results in loss of contact-dependent inhibition of growth and promotes anchorage-independent growth. Merlin loss enhances cell proliferation by increasing entry into the S-phase and cyclin E1 expression. Inactivation of merlin results in increased YAP expression and nuclear accumulation of YAP in these meningioma cell lines and in primary human meningioma tumors. Finally, we show that YAP suppression reverses the proliferation effects associated with merlin loss in meningiomas. Collectively, these data demonstrate that merlin regulates cell growth in a YAP-dependent manner in meningiomas and suggests that YAP is a compelling target for therapeutic inhibition of human meningioma tumor growth.

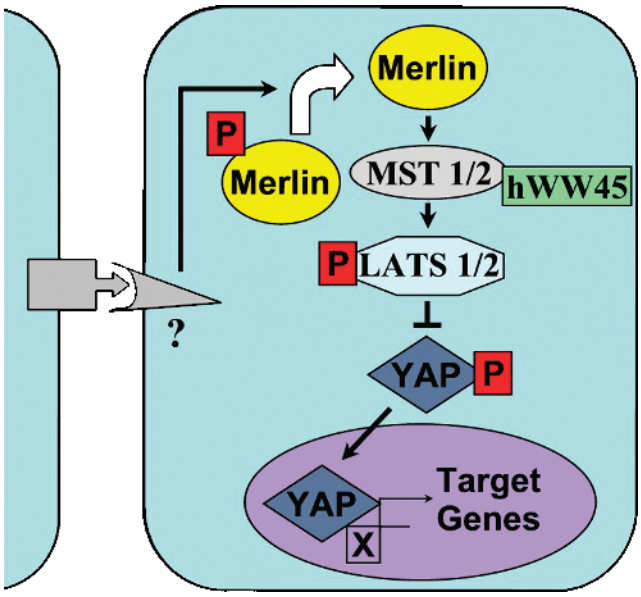


Figure 1. Schematic of the mammalian Hippo signaling pathway. The Hippo pathway is an evolutionary conserved cellular pathway that coordinately regulates cell proliferation and apoptosis. Merlin has been proposed to interact with unknown membrane proteins and transduce a signal that stimulates the phosphorylation of LATS1/2 by the serine/threonine kinases MST1/2 that interact with hWW45. LATS1/2 inhibits the transcriptional coactivator YAP resulting in suppressed expression of downstream target genes such as cyclins that are involved in cell growth and proliferation.

Materials and Methods

Tumor Samples, Cell Lines, and Culture

All human meningioma tumor samples were collected by the Neurological Surgery Tissue Bank using protocols approved by the University of California, San Francisco Committee on Human Research. Human meningioma cell lines used were KT21MG1 [27] and MENII-1. MENII-1 cells were isolated from a surgically resected grade II meningioma and were immortalized by the expression of telomerase and the human papillomavirus *E6/E7* genes as described earlier [28]. Human arachnoidal cells (AC1) were cultured by plating small fragments of surgically resected spinal arachnoid tissue on scores scratched on the bottom of six-well tissue culture plates. Within 7 days, cells with characteristic arachnoidal morphology growing as a monolayer of polygonal cells with large cytoplasmic arcs migrate out of these scores. The arachnoidal origins of these cells were verified by positive staining for vimentin and desmoplakin. Primary cultures of arachnoidal cells were immortalized by stable transfection with the human papillomavirus *E6/E7* oncogenes and telomerase as described earlier [28]. All cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and appropriate antibiotic selection markers.

Expression Constructs and Antibodies

The pSUPER.retro.neo-*NF2*-siRNA construct was generated by digesting the pSUPER.retro.neo vector (Oligoengine) with *Bgl*II and *Hind*III and ligating the annealed oligos (5′-gatccccGCAGCAAGCACAATACCATttcaagagaATGGTATTGTGCTTGCTGCttttta and 5′-agcttaaaaaGCAGCAAGCACAATACCATtctcttgaaATGGTATTGTGCTTGCTGCggg) that contain a 19-nucleotide *NF2* target sequence (in caps) using the strategy described earlier [29]. The pSUPER.retro.neo-YAP-siRNA construct was also generated in pSUPER.retro.neo using the strategy described above and a previously described YAP target sequence (CCAGAGAATCAGTCAGAGA) [30]. A nonspecific mammalian scramble sequence (Oligoengine, Seattle, WA) in pSUPER.retro.neo (pSUPER.retro.neo-Control) was used as a control for the development of stable cell lines. Wild type *NF2*, S518A *NF2*, and S518D *NF2* mutant constructs in pUHD10.3 have been previously described [7,31]. These three constructs were subcloned into pBABE-Hygro using standard techniques. Merlin polyclonal (A19, #sc-331) and monoclonal (B12, #sc-55575) antibodies and the cyclin E1 monoclonal antibody (13A3, #sc-56310) were from Santa Cruz Biotechnology, Santa Cruz, CA. The YAP monoclonal antibody (#4912) was from Cell Signaling (Danvers, MA), the cyclin D1 monoclonal antibody

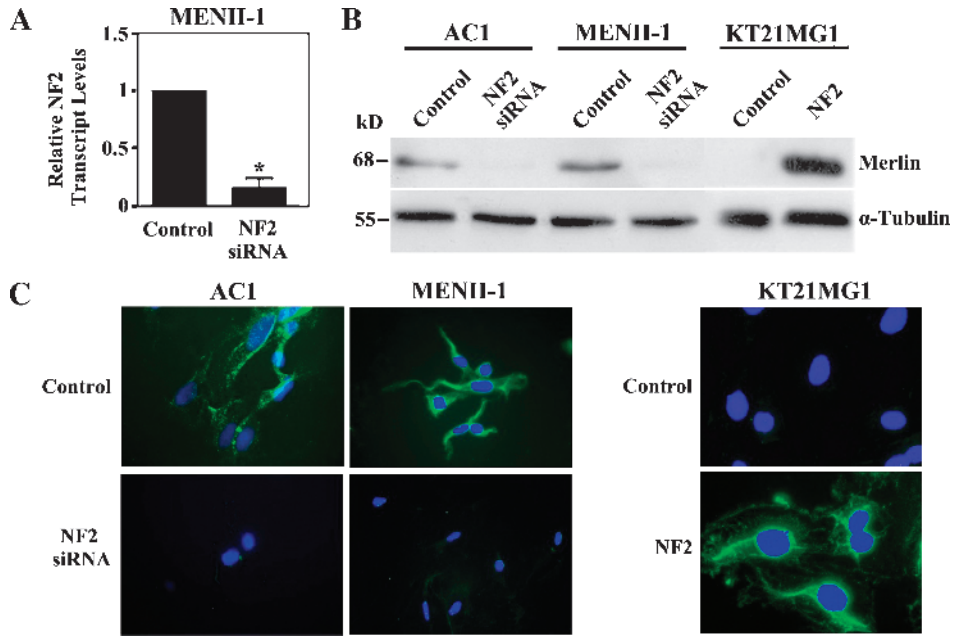


Figure 2. *In vitro* model system of merlin expression in human meningeal cells. Endogenous merlin was silenced in AC1 and MENII-1 cells using *NF2* specific siRNA. In parallel, merlin isoform 1 was exogenously expressed in KT21MG1 cells using retroviral mediated gene transfer. (A) *NF2* transcript levels were measured by quantitative PCR and showed a 5.6-fold reduction in MENII-1-*NF2*-siRNA cells compared with MENII-1-Control cells. Asterisk denotes statistical significance ($P < .05$). (B) Western blot analysis of cell lysates derived from AC1, MENII-1, and KT21MG1 stable cell populations was used to confirm loss or gain of merlin. Whereas merlin expression was observed in AC1-Control and MENII-1-Control cells, *NF2* siRNA abolished expression of merlin in AC1-*NF2*-siRNA and MENII-1-*NF2*-siRNA cells. In parallel, KT21MG1-Control cells lacked merlin, whereas KT21MG1-NF2 cells expressed wild type merlin. Levels of α-tubulin were determined in the same samples as a loading control. Immunoblot of one representative experiment of three with similar results is shown. (C) Immunofluorescence using the A19 polyclonal antibody against merlin revealed the presence of cytoplasmic staining in AC1-Control and MENII-1-Control cells and its absence in AC1-*NF2*-siRNA and MENII-1-*NF2*-siRNA cells. In contrast, KT21MG1-Control cells had no staining, whereas KT21MG1-*NF2* cells had cytoplasmic staining. Merlin immunolabeling is shown in green, and nuclear DAPI counterstaining is shown in blue.

(clone DCS-6) was from BD Pharmingen (Franklin Lakes, NJ), and the α-tubulin (#CP06) antibody was from Calbiochem (San Diego, CA).

Retroviral Infection and Selection of Stable Cell Populations

To stably suppress *NF2* in MENII-1 and AC1 cell lines, retroviral supernatants were generated by transfecting Phoenix A packaging cells with pSUPER.retro.neo-*NF2*-siRNA or pSUPER.retro.neo-Control using Lipofectamine 2000 Plus Reagent (Invitrogen, Carlsbad, CA). The 48-hour posttransfection supernatant was harvested, filtered, and used to infect MENII-1 and AC1 cell lines in the presence of 8 μg/ml polybrene. Stable cell populations were selected using 500 μg/ml G418. In parallel, stable cell populations expressing wild type or mutant *NF2* were generated by transfecting Phoenix A cells with the particular pBABE-Hygro construct and infecting KT21MG1 cells with the 48-hour posttransfection supernatant. Stable cell populations were selected using 200 μg/ml hygromycin. Empty pBABE-Hygro vector was used as a negative control.

Transient Suppression of YAP

Merlin-positive and -negative MENII-1 stable cell populations generated above were plated at 80% confluency in 100-mm dishes and transfected with 10 μg of pSUPER.retro.neo-YAP siRNA using Lipofectamine 2000 Plus Reagent (Invitrogen). Empty pSUPER.retro.neo vector was used as a negative control. The 72-hour posttransfection cells were subjected to flow cytometry to quantify bromodeoxyuridine (BrdU) uptake (described below).

Quantitative Polymerase Chain Reaction

Quantitative polymerase chain reaction (PCR) was performed in three independent experiments using cDNA templates with the I-cycler machine (Bio-Rad, Hercules, CA) and SYBR Green I (Molecular Probes, Eugene, OR) using PCR conditions and data analysis as described earlier [32]. Primers specific for GAPDH and actin were used to verify the integrity of the cDNA and to normalize cDNA yields. The primers used were: GAPDH, 5′-GGAAGCTTGTCATCAATGGAA and 5′-AAATGAGCCCCAGCCTTCTC; Actin, 5′-CAGGAGGAGCAATGATCTTG and 5′-ACTCTTCCAGCCTTCCTTCC; NF2, 5′-ACCGTTGCCTCCTGACATAC and 5′-TCGGAGTTCTCATTGTGCAG; YAP, 5′-GCAGTTGGGAGCTGTTTCTC and 5′-GCCATGTTGTTGTCTGATCG; cyclin E1, 5′-CCATCCTTCTCCACCAAAGA and 5′-TTTGATGCCATCCACAGAAA; cyclin D1, 5′-TGTTTGCAAGCAGGACTTTG and 5′-CCTTCCGGTGTGAAACATCT.

Western Blot Analysis

Total cell lysates were prepared either in buffer A (50 mM Tris-HCl, pH 7.5; 1 mM EDTA pH 8.0, 1% Triton) for merlin detection or in 1× SDS buffer following manufacturer's instructions (Cell

Signaling) for YAP and cyclin protein detection. Protein (50-200 μg) was resolved by electrophoresis for each sample and was transferred to a polyvinylidenefluoride membrane. Membranes were blocked in 5% low-fat dry milk in Tris-buffered saline–Tween 20 and incubated overnight at 4°C with either merlin A19 or B12 antibodies, or the YAP, cyclin E1, or cyclin D1 antibody, or α-tubulin. Incubation with horseradish peroxidase–conjugated goat antirabbit or antimouse immunoglobulin (Jackson Immunoresearch, West Grove, PA) was performed for 1 hour at room temperature. Bound antibody was visualized by chemiluminescence using the SuperSignal West Pico substrate (Pierce Chemical Co., Rockford, IL). The molecular weights were determined with the use of prestained protein ladders (BioRad, Hercules, CA, and Invitrogen). Films were scanned and exported as TIFF files.

Immunofluorescence Microscopy

Indirect immunofluorescence for merlin (A19 antibody or B12 antibody) and YAP was performed as described earlier [28]. Briefly, cells were fixed, permeabilized, blocked, and sequentially incubated with primary and secondary (Alexa 488 goat antirabbit IgG or Alexa 546 goat antimouse IgG) antibodies. Cells were mounted in DAPI mounting media, examined, and photographed with a microscope (Zeiss, Thornwood, NY).

Immunohistochemistry

Immunohistochemical staining was performed on 5-μm formalin-fixed, paraffin-embedded sections from meningioma tissue (8 primary tumors) and from a meningioma tissue microarray (29 primary tumors) for merlin (B12 antibody) and YAP as described earlier [33]. Slides were reviewed in consultation with a neuropathologist (S.R.V.) and included 33 WHO grade I and 4 WHO grade III meningiomas.

Growth Curves and Soft Agar Assay

Merlin-positive and -negative stable cell populations (20,000 cells) were plated in 24-well plates, and cells from three wells were counted at 3, 6, 12, 18, 25, and 30 days. To assess colony growth in soft agar, 50,000 cells were plated in Dulbecco's modified Eagle's medium in 0.4% low melting temperature agarose upon a layer of 0.8% agarose. After 8 weeks, colonies were stained with 0.005% crystal violet, and colonies larger than 100 μm in diameter were scored by counting under a microscope.

Flow Cytometry

Cells (70-80% confluent) were incubated with 1 mM BrdU for 3 hours at 37°C and processed using the fluorescein isothiocyanate BrdU Flow Kit (BD Biosciences, San Jose, CA) following manufacturer's instructions. Briefly, 1×10^6 trypsinized cells were fixed, permeabilized, and digested with DNAse. Cells were then stained with fluorescein isothiocyanate–conjugated anti-BrdU and 7-aminoactinomycin (7-AAD). Flow cytometry was performed on a Becton Dickinson FACSCalibur machine. For each experiment, 10,000 events were counted. Data acquisition was performed with the CellQuest software (BD Biosciences), and data were analyzed using Flow Jo v8.5.3.

Statistics

All data are expressed as mean ± SEM. GraphPad Prism version 4 was used for statistical analysis, consisting of unpaired *t* test and significant differences with a $P < .05$.

Results

Establishment of Matched NF2-Expressing and NF2-Deficient Human Arachnoidal and Meningioma Cell Lines

To develop a meningioma-specific NF2 *in vitro* model system, we determined the expression levels of endogenous merlin in normal human arachnoidal and meningioma cell lines (data not shown). On the basis of these results, we selected one arachnoidal cell line (AC1), one merlin-positive grade II meningioma cell line (MENII-1), and one merlin-negative grade III meningioma cell line (KT21MG1) for further analysis. Paired cell lines were generated by either suppressing *NF2* expression in AC1 and MENII-1 cells using RNA interference (siRNA) or by overexpressing the full-length human *NF2* cDNA (isoform 1, lacking exon 16 sequences) in KT21MG1 cells after retroviral-mediated gene transfer. Stable cell populations expressing either *NF2* siRNA (AC1-*NF2*-siRNA or MENII-1-*NF2*-siRNA) or a nonspecific target siRNA (AC1-Control or MENII-1-Control) were

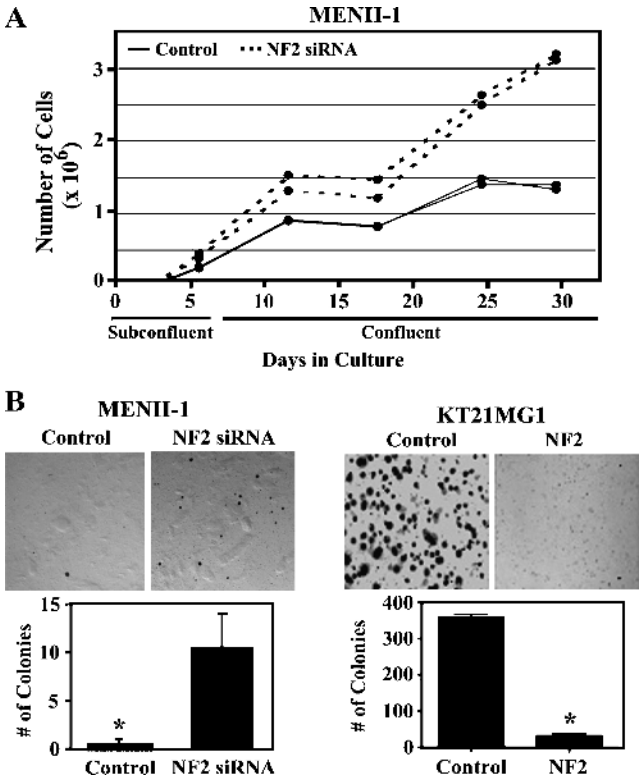
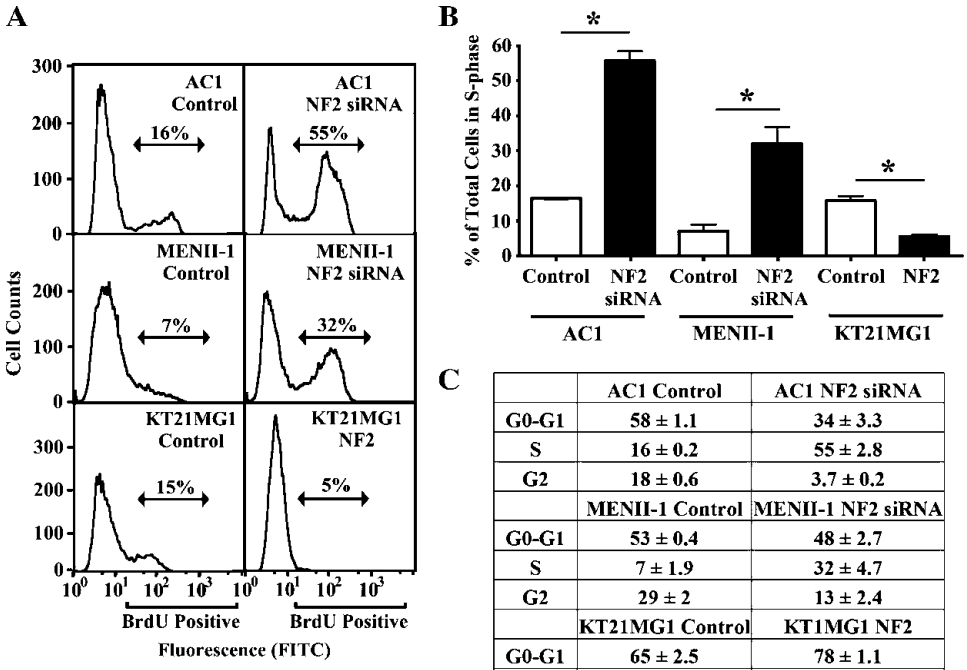


Figure 3. Suppression of merlin causes loss of contact-dependent inhibition of growth and promotes anchorage-independent growth. (A) Growth curves in the presence and absence of merlin expression. Cultures were subconfluent during the first 6 days. MENII-1-*NF2*-siRNA cells (*dotted lines*) continued growing after confluent conditions and have less contact-dependent inhibition of growth compared with MENII-1-Control cells (*solid lines*). Each line corresponds to representative cultures. (B) *NF2* suppression promotes anchorage-independent growth. Marked increased in colony formation in soft agar was observed in cells without merlin expression as MENII-1-*NF2*-siRNA and KT21MG1-Control cells compared with MENII-1-Control and KT21MG1-*NF2*, respectively. Representative images of the colonies formed (*upper panel*) and the mean number of colonies per well (*lower panel*) are shown. Error bars equal ±SE of three independent experiments. Asterisks denote statistical significance ($P < .05$).



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	AC1 Control	AC1 NF2 siRNA
G0-G1	58 ± 1.1	34 ± 3.3
S	16 ± 0.2	55 ± 2.8
G2	18 ± 0.6	3.7 ± 0.2
	MENII-1 Control	MENII-1 NF2 siRNA
G0-G1	53 ± 0.4	48 ± 2.7
S	7 ± 1.9	32 ± 4.7
G2	29 ± 2	13 ± 2.4
	KT21MG1 Control	KT1MG1 NF2
G0-G1	65 ± 2.5	78 ± 1.1
S	15 ± 1.2	5 ± 0.6
G2	7.6 ± 0.7	7 ± 0.07

Figure 4. Merlin loss enhances S-phase entry. AC1 and meningioma cell lines (MENII-1 and KT21MG1) were labeled with BrdU and 7-AAD to assess the cell cycle distribution of individual cells by flow cytometry. (A) Representative flow cytometric histograms indicate an increase in the percent of BrdU-positive cells in AC1-*NF2*-siRNA, MENII-1-*NF2*-siRNA, and KT21MG1-Control cells compared with AC1-Control, MENII-1-Control, and KT21MG1-*NF2* cells, respectively. (B) Bar graphs depict the percentage of cells in the S-phase of the cell cycle (BrdU-positive cells) averaged from three independent experiments. Error bars correspond to ±SE. Asterisks denote statistical significance using unpaired *t* test ($P < .05$). (C) Table shows the mean of the percentage of cells ±SE in each phase of the cell cycle from three independent experiments.

selected. *NF2* transcript levels were 5.6-fold lower in MENII-1-*NF2*-siRNA cells when compared with MENII-1-Control cells using quantitative PCR (Figure 2*A*). Similarly, merlin protein expression was undetectable in AC1-*NF2*-siRNA and MENII-1-*NF2*-siRNA cells by Western blot analysis and immunofluorescence (Figure 2, *B* and *C*). In parallel, stable cell populations expressing either exogenous merlin (KT21MG1-*NF2*) or empty vector (KT21MG1-Control) were selected. Expression of merlin in KT21MG1-*NF2* cells was confirmed by Western blot and immunofluorescence (Figure 2, *B* and *C*). In total, we have generated three separate human meningeal and meningioma cell lines that differ only in their expression of merlin for subsequent study.

Merlin Loss Increases S-Phase Entry, Cell Proliferation, and Promotes Anchorage-Independent Growth

Next, we assessed the effect of merlin loss on the growth properties of these human meningioma cell lines. MENII-1-*NF2*-siRNA cells exhibited a more pronounced loss of contact-dependent inhibition of growth compared with MENII-1-Control cells (Figure 3*A*). Merlin loss also promoted colony formation in soft agar. In these experiments, MENII-1-*NF2*-siRNA cells formed a greater number of colonies (10.6 ± 3.2) larger than 100 μm in diameter compared with MENII-1-Control cells (0.4 ± 0.4; $P = .01$; Figure 3*B*). Conversely, merlin expression in KT21MG1 cells significantly decreased the formation of colonies (32 ± 6.3) compared with merlin-negative KT21MG1 cells (361 ± 4.9; $P \leq .0001$; Figure 3*B*).

To determine the effect of merlin on cell cycle progression, we measured BrdU incorporation and total DNA content by flow cytometry. Loss of merlin in AC1 and MENII-1 cells resulted in a significant increase in the percentage of BrdU-positive cells, indicated by an increase in S-phase entry (Figure 4). This is consistent with the observed increase in proliferation in *NF2*-deficient cells. Conversely, expression of exogenous merlin in KT21MG1 cells induced G_0/G_1 arrest and a concomitant decrease in the S-phase cell population (Figure 4). These results demonstrate that merlin functions as a negative growth regulator for both nonneoplastic leptomeningeal cells and meningioma cells and establishes this system as a tractable experimental platform for examining growth regulatory pathways.

Merlin Loss Is Associated with an Increase in YAP Protein Expression

In *Drosophila*, merlin controls cell proliferation and apoptosis by signaling through the Hippo pathway and its effector protein Yorkie, the ortholog of YAP [16,18]. To determine whether merlin might regulate meningioma cell growth by modulating Hippo pathway signaling, we investigated whether changes in merlin expression were associated with altered levels of YAP using our human NF2 meningioma model system. Transcript levels of YAP were unaffected by merlin loss in MENII-1 cells (data not shown); however, YAP protein expression was elevated in arachnoidal and meningioma cells lacking merlin expression and decreased in KT21MG1 cells expressing wild type merlin (Figure 5*A*). These results suggest that merlin regulates YAP expression at the translational or posttranslational level.

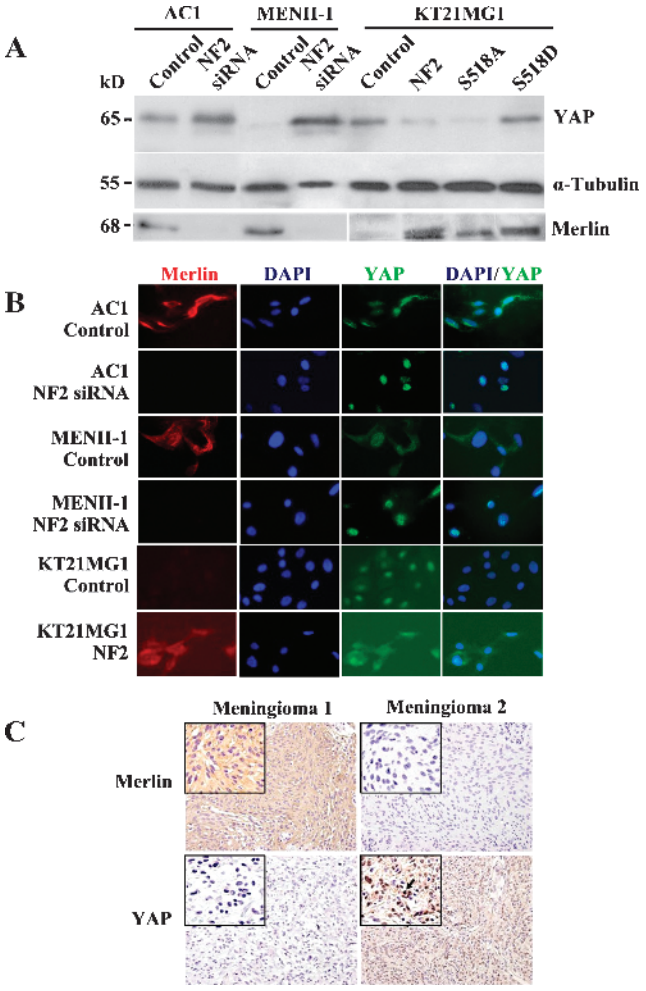


Figure 5. Protein levels of YAP are up-regulated and localized to the nucleus in *NF2*-deficient cells. (A) Total cell lysates were subjected to Western blot using a YAP- or merlin-specific antibody. Increased YAP protein expression was observed when *NF2* was suppressed in AC1 and MENII-1 cells compared with controls. Conversely, exogenous expression of merlin decreased YAP in KT21MG1 cells compared with controls. Expression of a nonphosphorylated, active merlin (S518A *NF2*) was also associated with lower levels of YAP compared with the expression of pseudophosphorylated inactive merlin (S518D *NF2*). Levels of α-tubulin were determined in the same samples as loading control. Results were reproduced in three independent experiments. (B) YAP was translocated to the nucleus in merlin-deficient cells. Immunofluorescence staining was used to show that YAP was localized to the nucleus in AC1-*NF2*-siRNA, MENII-1-*NF2*-siRNA, and KT21MG1-Control cells. In contrast, YAP was primarily cytoplasmic in AC1-Control, MENII-1-Control, and KT21MG1-*NF2* cells. Merlin immunolabeling is shown in red; YAP staining is shown in green; and nuclear DAPI counterstaining is shown in blue. (C) *In situ* immunostaining of merlin and YAP in serial sections of primary human meningioma tumors. We surveyed 37 primary meningiomas by immunohistochemistry. YAP expression was minimal to absent in 95% of merlin-positive meningiomas (a representative tumor is shown here as *Meningioma 1*). In contrast, YAP was expressed and localized to the nucleus in 92% of merlin-negative meningiomas (a representative tumor is shown here as *Meningioma 2*). Arrow depicts an example of YAP nuclear localization. Insets show images at higher magnification.

Merlin becomes active after dephosphorylation of the conserved C-terminal serine 518 (S518) residue [31,34]. Previous studies have shown that merlin mutants in which this phosphorylatable residue is changed to alanine (S518A) are constitutively nonphosphorylated and active, whereas those containing aspartic acid (S518D) are pseudophosphorylated and nonfunctional as negative growth regulators [31,34]. Using these mutants, we assessed the effect of merlin phosphorylation status on YAP protein levels in KT21MG1 human meningioma cells. YAP protein levels were markedly up-regulated in KT21MG1 cells expressing the S518D inactive mutant compared with those with active merlin S518A expression (Figure 5*A*), suggesting that active and functional merlin was required to inhibit YAP.

YAP has been reported to shuttle between the cytoplasm and the nucleus where it can function as a transcriptional coactivator [35]. To determine whether merlin regulates the subcellular localization of YAP, we used fluorescence immunocytochemistry to examine *NF2*-deficient and *NF2*-expressing AC1 and MENII-1 cells. YAP was preferentially expressed in the cytoplasm of AC1-Control and MENII-1-Control cells. In contrast, YAP was localized in the nucleus in AC1-*NF2*-siRNA and MENII-1-*NF2*-siRNA cells (Figure 5*B*). Collectively, these data indicate that merlin regulates YAP protein expression and YAP nuclear localization.

To assess whether a similar association exists between merlin and YAP in human meningioma tumors, we surveyed 37 sporadic primary meningioma tumors by immunohistochemistry. After immunostaining with a merlin-specific monoclonal antibody, meningiomas were classified as either merlin-positive, if tumors exhibited any positive immunoreactivity, or merlin-negative, if tumors had no immunoreactivity. We then assessed protein levels of YAP in adjacent serial sections. 13 (92%) of 14 merlin-negative meningiomas exhibited strong nuclear YAP immunoreactivity. In contrast, 22 (95%) of 23 merlin-positive meningiomas had weak to no YAP immunoreactivity (Figure 5*C*). These results further support the *in vitro* results demonstrating that merlin regulates YAP protein levels *in vivo*.

Merlin Loss Is Associated with an Increase in Protein Levels of Cyclin E1

Previous studies have identified cyclin E1 as a transcriptional target of the Hippo pathway in *Drosophila* [36–38] and an essential regulator of progression from G_1 to S cell cycle progression in mammalian cells [39,40]. Also, merlin has been reported to inhibit cell proliferation by repressing cyclin D1 in human mesothelioma cells [41]. To determine whether merlin regulates cyclin E1 and/or cyclin D1 levels in human meningioma cells, we measured cyclin E1 and D1 RNA and protein expression by quantitative PCR and Western blot analysis, respectively. Cyclin E1 transcript levels were at least 2.5-fold higher in MENII-1-*NF2*-siRNA cells compared with MENII-1-Control cells, whereas cyclin D1 transcript levels were the same in MENII-1-*NF2*-siRNA and MENII-1-Control cells (Figure 6*A*). In addition, cyclin E1 protein levels were elevated in MENII-1-*NF2*-siRNA and AC1-*NF2*-siRNA cells compared with MENII-1-Control and AC1-Control cells, respectively (Figure 6*B*). Conversely, exogenous expression of merlin in NF2-deficient KT21MG1 cells resulted in decreased cyclin E1 protein levels. In contrast, cyclin D1 protein levels were unaffected by the absence or presence of merlin in both MENII-1 and KT21MG1 cells (Figure 6*C*). These data suggest that merlin likely regulates cell

growth by modulating cyclin E1 expression at the transcriptional level in meningiomas.

YAP Reduction Reverses the Growth Phenotype Associated with Merlin Loss

Lastly, to determine whether YAP expression is necessary for the enhanced S-phase entry induced by merlin loss in meningioma cells, we transiently depleted YAP using RNA interference in MENII-1-Control and MENII-1-*NF2*-siRNA cells (Figure 7). Reduced YAP expression in *NF2*-deficient MENII-1 meningioma cells caused a ~50% decrease in the percentage of cells in S-phase (9 ± 1.2) compared with mock-transfected cells (20 ± 0.2; $P = .001$; Figure 7, *B* and *C*). In contrast, YAP siRNA treatment had a minor effect on MENII-1-Control cells (~20% reduction; $P = .08$; Figure 7, *B* and *C*). Collectively, these experiments demonstrate that YAP transduces the merlin growth regulatory signal in meningiomas and that the Hippo growth control pathway is responsible for merlin tumor suppressor function in this tumor type.

Discussion

The Hippo signaling pathway is emerging as an evolutionarily conserved mechanism that controls organ size and growth relevant to tumorigenesis [18,37]. In this study, we investigated the functional association between *NF2* gene expression and the downstream effector of the Hippo pathway in human meningiomas. We provide several lines of converging and complementary evidence that merlin functions through YAP in meningiomas. First, using paired meningioma cell lines differing only in *NF2* expression, we show that YAP expression is increased in a dose-response manner upon merlin loss. This regulation occurs at the translational or posttranslational level and results in YAP nuclear localization. Second, the relationship between merlin expression and YAP nuclear localization is also observed in human surgical meningioma specimens *in vivo*. These observations are consistent with findings made in other solid cancers in which Hippo signaling is deregulated [42]. Third, suppressed YAP expression in merlin-deficient meningioma cells attenuates the cell growth and S-phase cell cycle progression associated with merlin loss. To the best of our knowledge, these results represent the first demonstration that merlin regulates cell growth in human meningioma cells by suppressing YAP, the main Hippo pathway effector protein.

YAP has previously been implicated in other human cancers, including pancreatic ductal adenocarcinoma, and has been shown to function as an oncogene that induces epithelial transformation of mouse mammary cells [26,43]. Nuclear localization of YAP is dependent on the phosphorylation status of a conserved Ser residue and is necessary for its cotranscriptional activator function [35,44]. YAP associates with multiple transcription factors in the nucleus such as p73 and TEAD/TEF [44,45]. ErbB4 receptors have been reported to recruit YAP and relocate to the nucleus to regulate transcription [46,47]. In contrast, LATS1 inactivates YAP oncogenic function by sequestering YAP in the cytoplasm and, consequently, suppressing its transcriptional regulation of cellular genes [35]. Our findings are in accordance with these and suggest that the tumorigenic behavior of meningioma cells is driven in part by YAP nuclear localization and the transcription of genes involved in increased proliferation.

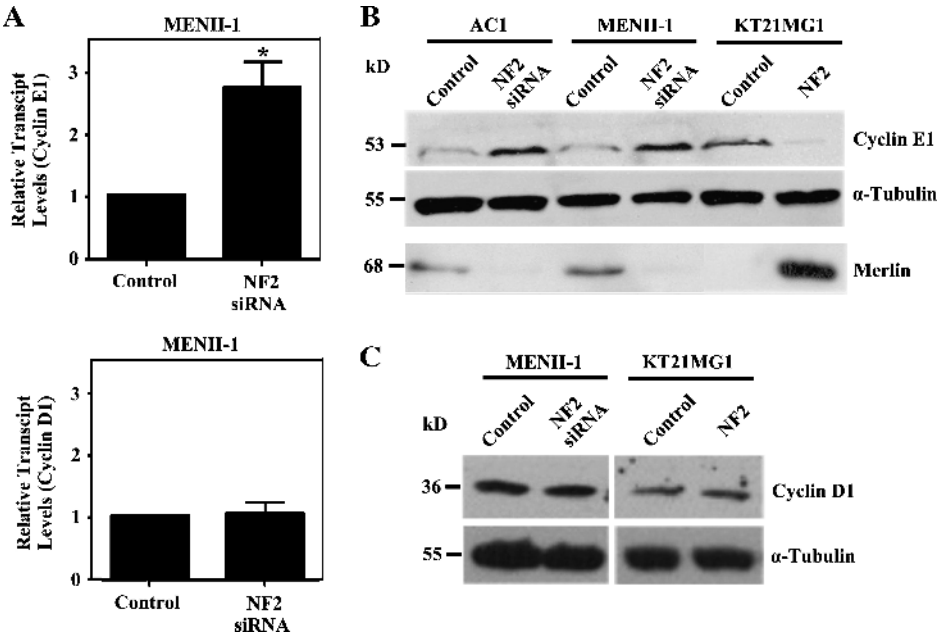
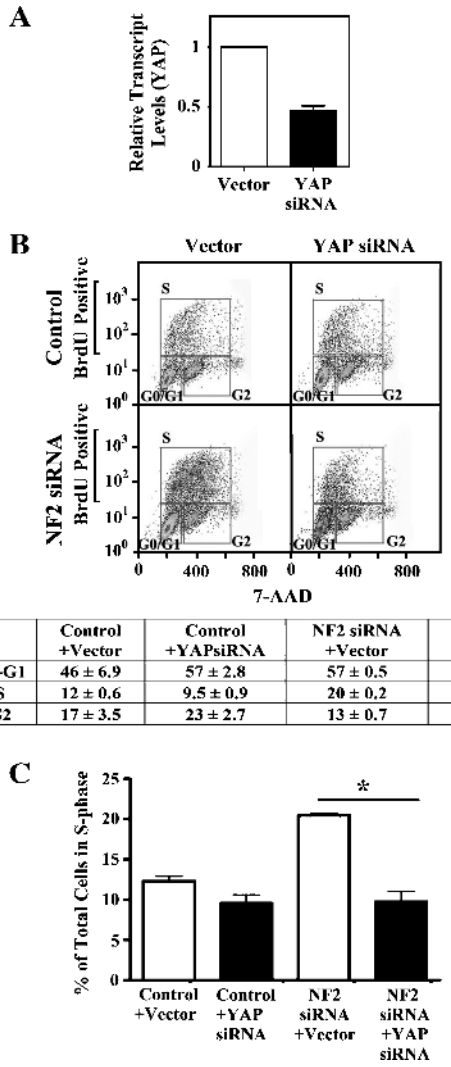


Figure 6. Cyclin E1 and cyclin D1 expression was increased in merlin-deficient cells. (A) Transcript levels of cyclin E1 and cyclin D1 were measured in MENII-1 cells using quantitative PCR. At least a 2.5-fold increase in the transcript levels of cyclin E1 was seen in MENII-1-*NF2*-siRNA cells compared with MENII-1-Control cells, whereas transcript levels of cyclin D1 were unchanged. Asterisk denotes statistical significance ($P < .05$). (B) Western blot analysis of cell lysates derived from AC1, MENII-1, and KT21MG1 stable cells was used to show that protein levels of cyclin E1 were increased in the absence of merlin. Levels of α-tubulin were determined in the same samples as a loading control. (C) Western blot analysis of cell lysates derived from MENII-1 and KT21MG1 stable cells was used to show that protein levels of cyclin D1 were unchanged in the absence of merlin. Levels of α-tubulin were determined in the same samples as a loading control.



	Control +Vector	Control +YAPsiRNA	NF2 siRNA +Vector	NF2siRNA +YAPsiRNA
G0-G1	46 ± 6.9	57 ± 2.8	57 ± 0.5	66 ± 2.3
S	12 ± 0.6	9.5 ± 0.9	20 ± 0.2	9 ± 1.2
G2	17 ± 3.5	23 ± 2.7	13 ± 0.7	17 ± 1.6

Figure 7. Down-regulation of YAP decreased proliferation in merlin-deficient cells. MENII-1-Control and MENII-1-*NF2*-siRNA cells were transiently transfected with YAP-specific siRNA or empty vector. (A) YAP transcript levels were measured in MENII-1-Control cells using quantitative PCR. The YAP-specific siRNA caused a 50% reduction in YAP transcript levels compared with controls in MENII-1 cells. (B, C) BrdU incorporation and 7-AAD staining were measured by flow cytometry. (B) One representative experiment shows the distribution of cells in G_0-G_1, S, and G_2 phases of the cell cycle. The table below shows the mean of the percentage of cells ±SE in each phase of the cell cycle from three independent experiments. (C) Bar graphs depict the percentage of cells in the S-phase of the cell cycle (BrdU-positive cells) averaged from three independent experiments. Error bars correspond to ±SE. Suppression of YAP decreased the percentage of cells in S-phase in MENII-1-*NF2*-siRNA cells to levels similar to MENII-1-Control cells.

We demonstrate that merlin controls the cell cycle in meningiomas. Merlin suppression results in increased S-phase entry, and merlin expression resulted in G_0/G_1 arrest. Similar results were obtained in mesotheliomas [41] and schwannomas [48]. These results provide strong evidence that merlin functions as a tumor suppressor by controlling the G_0/G_1– to S-phase checkpoint of the cell cycle. Cyclin E1 is thought to be essential for this cell cycle transition in humans [39,40] and has been identified as a downstream target of Yorkie in *Drosophila* [36–38]. In contrast, cyclin E1 was not induced after overexpression of YAP in mammary epithelial cells [26]. Instead, it has been shown that cyclin D1 is regulated by YAP in mouse intestine [25] and by merlin in human mesotheliomas [41]. Our experiments show that merlin regulates cyclin E1 but not cyclin D1 in human meningiomas. These differences most likely represent cell type–specific differences in gene expression. Further studies are needed to determine whether merlin regulates cyclin E1 by signaling through YAP.

The fact that NF2 patients develop only certain CNS tumors also emphasizes the cell type–specific tumorigenic effects of merlin. Merlin loss results specifically in aberrant growth of Schwann and meningeal cell types. However, not all meningiomas have *NF2* loss, suggesting that other mechanisms that do not involve the Hippo pathway result in meningioma formation. Alternatively, it remains to be elucidated whether other components of the Hippo pathway, downstream of merlin, are possibly deregulated in these tumors resulting in the same phenotype as merlin loss.

Treatment strategies for NF2 and sporadic meningioma patients are restricted to traditional forms of cancer therapy such as surgery and radiation therapy. These options are sometimes insufficient because of the location of these tumors, the recurrence despite therapies and the occurrence of multiple tumors. Although receptor tyrosine kinases inhibitors are emerging as pathway targets in other CNS tumors such as glioblastomas [49], targeted therapies have not yet been proposed for meningioma patients. This is mainly because of a lack of knowledge regarding relevant signaling pathways. Our results argue that YAP is an attractive candidate as a key mediator of *NF2* growth regulation and tumorigenesis in meningioma, making it a potential target for the development of therapies for NF2 and meningioma patients.

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